Characterization of Transgenic Rice Seedlings Overexpressing OsWRKY11 Under the Control of HSP101 Promoter

	WU　　XIAOLAN
氏名（本籍地）	呉　　暁　嵐
学位の種類	博士（農学）
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論文題目	Characterization of Transgenic Rice Seedlings Overexpressing *OsWRKY11* Under the Control of *HSP101* Promoter （*OsWRKY11* を *HSP101* プロモーターにより過剰発現させた形質転換イネの解析）
博士論文審査委員	（主査）教　授　鳥　山　欽　哉 教　授　國　分　牧　衛 准教授　伊　藤　幸　博 准教授　北　柴　大　泰

論 文 内 容 要 旨

Introduction

Drought stress is known to be one of the major causes of reduced crop yield, and great efforts have been made to breed drought tolerant crop varieties. Many transgenic approaches have been carried out to increase biotic and abiotic stress tolerance. One successful approach to increase abiotic stress tolerance is to employ overexpression of certain stress-inducible transcription factors. However, employment of a constitutive promoter to drive genes for transcription factors may cause growth retardation under unstressed control conditions in transgenic plants. It has been reported that the stress-inducible promoter minimizes this negative effect on plant growth. It is, therefore, desirable to use stress-inducible promoters to produce stress tolerant plants.

WRKY genes encode a large group of transcription factors. There are over 100 *WRKY* genes in rice. This family is defined by a domain of 60 amino acids containing the amino acid sequence WRKY at its amino-terminal end and a putative zinc finger motif at its carboxy-terminal end. *WRKY* genes are known to participate in various developmental and physiological programs, including disease-resistance, senescence, biotic and abiotic stress responses, and growth and developmental processes. However, no information is available regarding the relationship between overexpression of *WRKY* genes and heat and drought tolerance.

OsWRKY11 (DDBJ accession no. AK108745) has been identified to be one of the transcription factors showing enhanced expression by heat treatment, drought treatment and by combined heat/drought treatment (Shiroto et al. 2004). Transgenic rice plants (*Oryza sativa* L. cv. Sasanishiki) with *OsWRKY11* cDNA or *GUS* gene under the control of the *HSP101* promoter have been produced in our laboratory (Shiroto et al. 2004), but further characterization of these plants has not yet been achieved. The promoter of heat shock protein gene, *HSP101,* was employed, because it was expected that this promoter was stress-inducible and could be used to minimize the possible deleterious effects of *OsWRKY11* expression under unstressed conditions.

In my study, to determine if overexpression of *OsWRKY11* confers heat and drought tolerance, I first examined activation of *HSP101* promoter by heat and drought stress (Chapter 1) and the vegetative phenotype of transgenic plants overexpressing *OsWRKY11* (Chapter 2). Then I evaluated drought tolerance by investigating the survival of the green parts of plants after heat and drought treatment, and water loss of

detached aerial parts during desiccation (Chapter 3). Enhanced drought tolerance and increased amounts of osmoprotectants were observed. Because overexpression of some other *WRKY* genes has been reported to confer biotic stress-tolerance, I also evaluated the degree of blast disease resistance (Chapter 4). Through microarray analysis of gene expression in the transgenic plants overexpressing *OsWRKY11*, I observed two genes possibly related to the phenotype (Chapter 5). A novel regulatory network of abiotic-stress tolerance via *OsWRKY11* was discussed.

Chapter 1 Activation of *HSP101* promoter by heat stress

Transgenic rice plants with *HSP101* promoter::*GUS* (Fig.1) were grown at 22/18°C (12/12 h), and excised leaf blades were subjected to histochemical GUS assay. Incubation of the leaf segments at 37°C or 42°C for 1 hr induced a high level of *GUS* expression, while no *GUS* expression was detected at room temperature (Fig. 2). The GUS activity under other stresses, i.e., cold (4°C), 250 mM NaCl or desiccation for 1hr was not detected. Thus the heat shock activation of the *HSP101* promoter was confirmed.

Transgenic lines (i.e., ox2 and ox3) with a single copy of the T-DNA containing *HSP101* promoter::*OsWRKY11* (Fig.1) were selected. T_2 plants homozygous for the T-DNA were used in this study. A transgenic line, g8, which contained a single copy of *HSP101* promoter::*GUS*, was used as a negative control. RT-PCR and real-time quantitative RT-PCR analysis in leaves of ox2 and ox3 seedlings demonstrated that overexpression of *OsWRKY11* was induced by heat treatment, but it was not detectable under unstressed normal conditions (Fig. 3).

Chapter 2 Growth defects of transgenic plants overexpressing *OsWRKY11*

Four lines of transgenic rice with *HSP101* promoter::*OsWRKY11* were used in this study (ox2, ox3, o4, and ox5; Table 1). Under normal growth conditions, three types of phenotypes were observed: dwarf with bent leaves, normal plant length with bent leaves and normal plant length with normal leaves (Table 1). Some of the ox4 and ox5 plants showed dwarf phenotype. The dwarf phenotype and bent leaves were manifested when the plants were subjected to heat treatment at 37/22°C (12/12 h) (Fig. 4). These results

suggested that introduction of *HSP101* promoter::*OsWRKY11* might have had some deleterious effects on plant growth. Under normal growth conditions, the seed set percentage was 36.7 ± 5.8 for ox2 and 98.0 ± 0.8 for ox3, while it was 99.8 ± 0.5 for WT.

Chapter 3 Enhanced drought tolerance of transgenic seedlings

Two-week-old seedlings grown at 22/18°C (12/12 h) were pre-treated for 2-weeks at 37/22°C for the induction of *OsWRKY11* expression. Then the plants were exposed to combined heat/drought treatment at 37/22°C without a water supply for 2 days. The *OsWRKY11* transgenic lines ox2 and ox3 showed weaker leaf-wilting phenotype (Fig. 5). After recovery for 2 weeks, the areal parts were separated into green living parts and non-green dead parts, and each DW was determined. The transgenic lines ox2 and ox3 showed a high level of surviving green rate (Fig. 6). The combined heat/drought treatment for 2.5 days demonstrated that the *OsWRKY11* transgenic lines survived, whereas WT plants died after recovery at 22/18°C for 2 weeks (Fig. 7). These results demonstrated that the *OsWRKY11*-overexpressed lines ox2 and ox3 gained significant drought tolerance.

Desiccation tolerance was evaluated by investigating the time courses of water loss in detached aerial parts. The water loss in ox2 and ox3 was significantly slower than that in WT and g8 (Fig. 8). Comparison of the ratio between the time of 20% water loss of plants with the heat pretreatment and that with the no-heat pretreatment demonstrated that the heat pretreatment enhanced desiccation tolerance, especially in the ox2 and ox3 plants (Table 2).

Sucrose, glucose, fructose and raffinose were more highly accumulated in plants with heat treatment than in plants with no heat treatment. (Fig. 9). The content of four sugars was higher in the *OsWRKY11*-overexpressed plants than in WT even with no-heat treatment. This suggests that sucrose, glucose, fructose and raffinose acted as osmoprotectants in rice plants. In particular, raffinose accumulated at a significantly higher level in the heat-treated ox2 and ox3 plants, while the unstressed WT plants had no detectable amount of raffinose, suggesting an important role of raffinose accumulation in the desiccation tolerance of the *OsWRKY11*-overexpressed plants.

Chapter 4 Effects on blast resistance

Since *OsWRKY11* expression has been reported to be increased in an incompatible interaction between rice and rice blast fungus (*Magnaporthe grisea*), the transgenic plants ox2 and ox3 were expected to gain tolerance to such biotic stresses. Conidia of blast fungus (race 007) suspended at a density of 10^5 / ml was sprayed onto rice plants. The transgenic *OsWRKY11* lines showed no decreases in susceptible-type lesions compared with WT plants (Fig. 10), indicating no effect of *OsWRKY11* overexpression on blast resistance.

Chapter 5 Up- or down-regulated genes revealed by Microarray analysis

To determine the kind of genes which are up- or down-regulated by *OsWRKY11* overexpression, gene expression profiles were compared between ox3 and WT with or without heat treatment at 37/22°C for 2 weeks using 44K Agilent microarray. After heat treatment, 840 genes showed changes of 2-fold or higher ($p<0.05$) (Fig. 11). The up-regulated genes did not include the well-known ABA-induced genes (Table 3), indicating that ABA drought tolerant networks were not likely involved in conferring enhanced heat and drought tolerance in the *OsWRKY11*-overexpressed plants. Up-regulated genes included a gene for raffinose synthase (AK120944), which was 6.2-fold up-regulated. This gene likely functions to accumulate raffinose and confer desiccation tolerance in the transgenic ox3.

In contrast, *ent*-kaurene oxidase, which functions in gibberellin synthesis, was down-regulated (Table 4). This accounts for the fact that transgenic ox3 shows dwarf phenotype after heat treatment. *OsWRKY11* is considered to be involved in activating production of osmoprotectants while suppressing gibberellin synthesis.

Conclusion

My study proposes an ABA-independent novel pathway of drought stress response and tolerance in which *OsWRKY11* plays a role through accumulation of osmoprotectants (Fig. 12).

Drought and heat are major abiotic stresses on crop production associated with global warming. Heat stress, however, often precedes drought stress. The *HSP101* promoter will be useful to confer combined heat/drought tolerance in natural conditions,

minimizing growth defects in unstressed conditions. My study demonstrates that *OsWRKY11* together with the *HSP101* promoter holds promising utility in improving drought tolerance in rice.

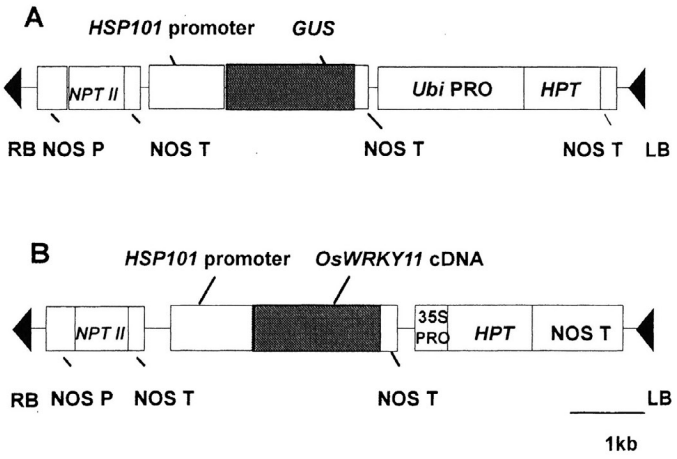

Fig. 1. The T-DNA region introduced into rice plants with *HSP101* promoter::*GUS* (A) and *HSP101* promoter::*OsWRKY11* (B). *HSP101* promoter was amplified from rice genome based on the sequence of DDBJ accession no. AJ316025. *OsWRKY11* cDNA was a full length cDNA (accession no. AK108745). LB, left border; NOS P, Nopaline synthase promoter; NPTII, Neomycin phosphotransferase; NOS T, 3' signal of nopaline synthase terminator; 35S PRO, Cauliflower Mosaic Virus 35S promoter; HPT, hygromycin phosphotransferase.

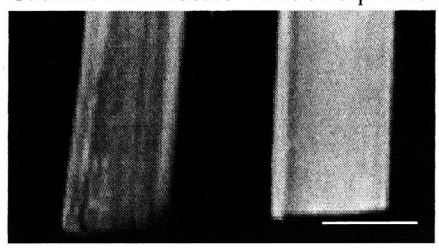

Fig. 2. GUS assay of leaf blade in transgenic plants with *HSP101* promoter::*GUS* with or without heat shock. Strong GUS activity, blue staining, was observed after heat shock at 37°C for 1 hr (leftt) but not evident at control condition without heat shock (right). Bar=1cm

Table 1 Phenotype of mature transgenic plants (T_1) segregating for the introduced *OsWRKY11*.

Transgenic line	ox2	ox3	ox4	ox5	WT
Copy number of the transgene	1	1	2	4	0
Total Number of plants examined	5	24	6	4	4
Number of plants without the transgene	1	5	2	1	4
Number of dwarf plants (plant length<85cm)	0	0	0	0	0
Number of normal plant length (>94 cm) with bent leaves	0	0	0	0	0
Number of normal plant length (>94 cm) with normal leaves	1	5	2	1	4
Number of plants with the transgene	4	19	4	3	0
Number of dwarf plants (plant length<85cm)	0	0	2	1	0
Number of normal plant length (>94 cm) with bent leaves	4	16	0	0	0
Number of normal plant length (>94 cm) with normal leaves	0	3	2	2	0

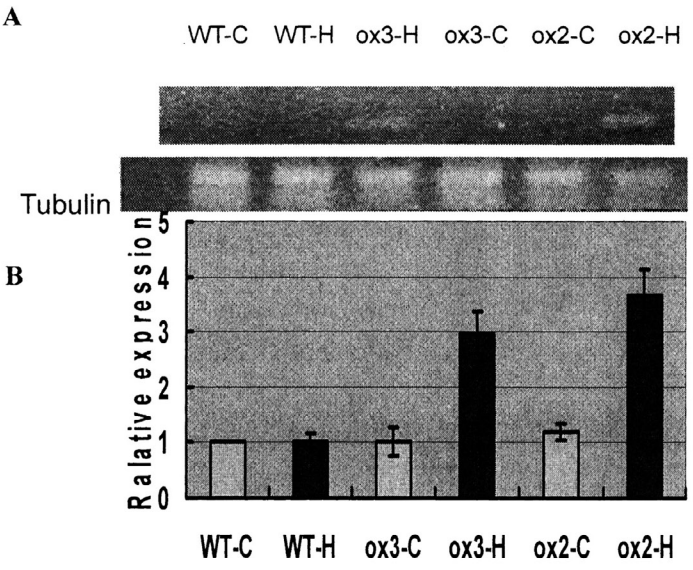

Fig.3 The gene expression of *HSP101* promoter::*OsWRKY11*. A, RT-PCR analysis of *HSP101* promoter::*OsWRKY11* gene expression with no-heat treatment (C) at 22/18°C (12/12 h) or plants with heat treatment (H) at 37/22°C (12/12 h) for 2 weeks. B, Relative *OsWRKY11* expression level compared to that of *tubulin alpha-1* chain detected by real-time quantitative RT-PCR in plants with no-heat treatment (C) at 22/18°C (12/12 h) or plants with heat treatment (H) at 37/22°C (12/12 h) for 2 weeks. Value indicates relative expression level against that of WT-C in three biological replicates from cDNA prepared from leaf blades. Overexpression of Os*WRKY11* was induced by heat treatment. The expression level after no-heat treatment was the same as that in WT. ox2 and ox3, T_2 plants with *HSP101* promoter::*OsWRKY11; g8, HSP101* promoter::*GUS*.

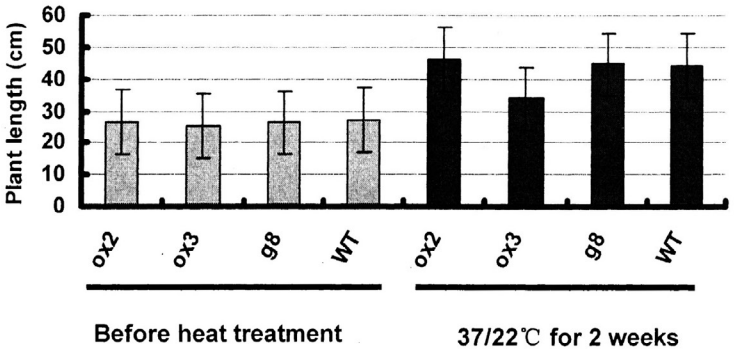

Fig. 4 Plant lengths of 2-week-old seedlings before or after heat treatment for 2 weeks. Transgenic line ox3 showed a dwarf phenotype after heat shock. ox2 and ox3, T_2 plants with *HSP101* promoter::*OsWRKY11; g8, HSP101* promoter::*GUS*.

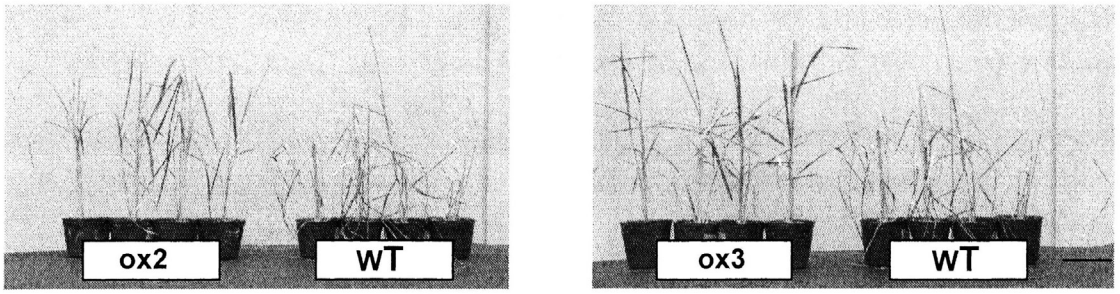

Fig. 5 Photographs of Os*WRKY11* transgenic lines ox2 and ox3, and WT after combined heat/drought treatment at 37/22°C (12/12 h) without a water supply. The photographs were taken after combined heat/drought treatment for 2 days. The *OsWRKY11* transgenic lines ox2 and ox3 show weaker leaf-wilting phenotype. Bar = 5 cm.

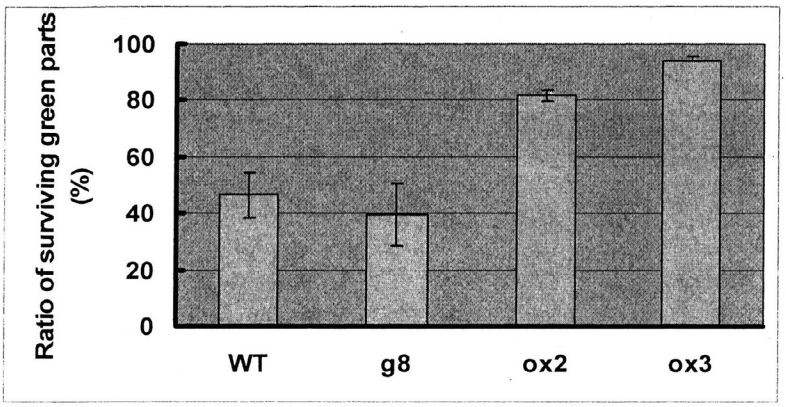

Fig. 6 Survival of green parts in seedlings after recovery for 2 weeks from exposure to combined heat/drought treatment at 37/22°C (12/12 h) for 2 days without a water supply. Values (mean±SD) indicate the percentage of surviving green parts calculated as the (DW of green parts / DW of green parts plus dead parts) x 100 (n=4). The *OsWRKY11* transgenic lines ox2 and ox3 conferred significant drought tolerance compared to WT and g8, plants with *HSP101* promoter::*GUS*.

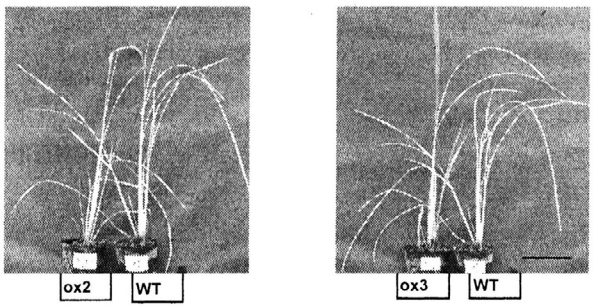

Fig. 7 Photographs of Os*WRKY11* transgenic lines ox2 and ox3, and WT after combined heat/drought treatment at 37/22°C (12/12 h) without a water supply. The photographs were taken after combined heat/drought treatment for 2.5 days. The *OsWRKY11*transgenic lines ox2 and ox3 survived, whereas WT plants died after recovery at 22/18°C for 2 weeks. Bar = 5 cm.

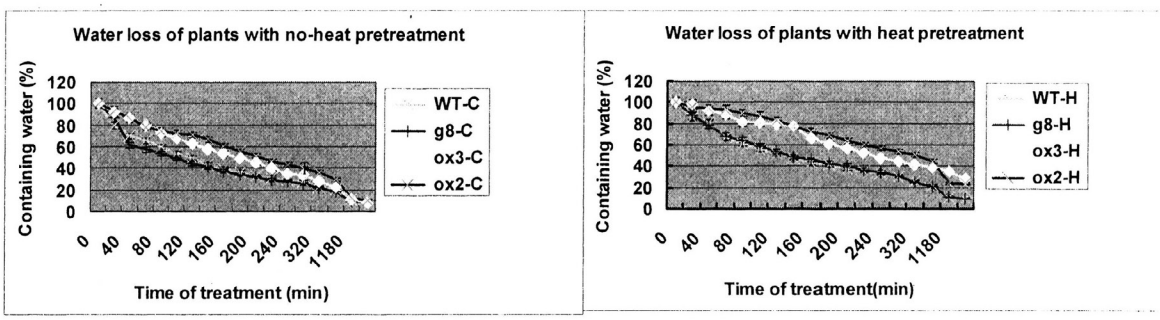

Fig. 8 The time courses of water loss in detached aerial parts. The water loss in ox2 and ox3 was slower than that in WT and g8. The water loss of plants with heat pretreatment (H) was slower than that of with no-heat pretreatment (C). ox2 and ox3, T_2 plants with *HSP101* promoter::*OsWRKY11; g8, HSP101* promoter::*GUS*. Values (mean±SD) indicate relative water content calculated as $[(FW_i - DW)/(FW_0 - DW)] \times 100$, whereas FW_i and FW_0 are fresh weight for any given interval and original fresh weight, respectively, and DW is dry weight (n=15 for ox2, ox3 and WT. n=14 for g8).

Table 2 Comparison of the time for 20% water loss of plants with the heat pretreatment (H) and those with the no-heat pretreatment (C). Heat pretreatment enhanced the desiccation tolerance, especially in the ox2 and ox3 plants.

	Time for 20% water loss (min)		
	Heat pretreatment (H)	**No-heat pretreatment (C)**	**Ratio (H / C)**
WT	52. 7 ± 5.7	37.7 ± 4.7	1.4
g8	51.0 ± 11.4	39.0 ± 3.5	1.3
ox3	120. 7 ± 1.5	66.3 ± 3.8	1.8
ox2	128.0 ± 11.4	67.3 ± 2.9	1.9

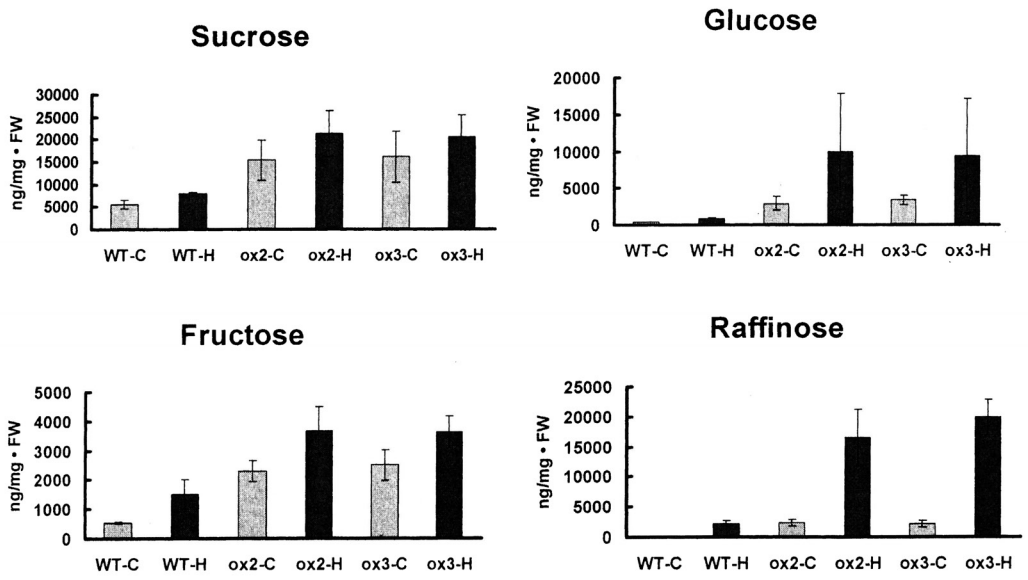

Fig. 9 Sugar contents of the transgenic rice plants overexpressing the *OsWRKY11* gene ox2 and ox3 and WT. Sugars were extracted with 80% ethanol from leaves and were analysed using Saccharose/ D-Glucose/ D-Fructose kit and Raffinose kit (Roche). Sucrose, glucose, fructose and raffinose accumulated more highly in plants with heat treatment (H) than in those with no heat treatment (C). The content of four sugars was higher in transgenic plants than in WT even with no-heat treatment. While the unstressed WT plants had no detectable amount of raffinose, raffinose accumulated at a significantly higher level in heat treatment ox2-H and ox3-H plants.

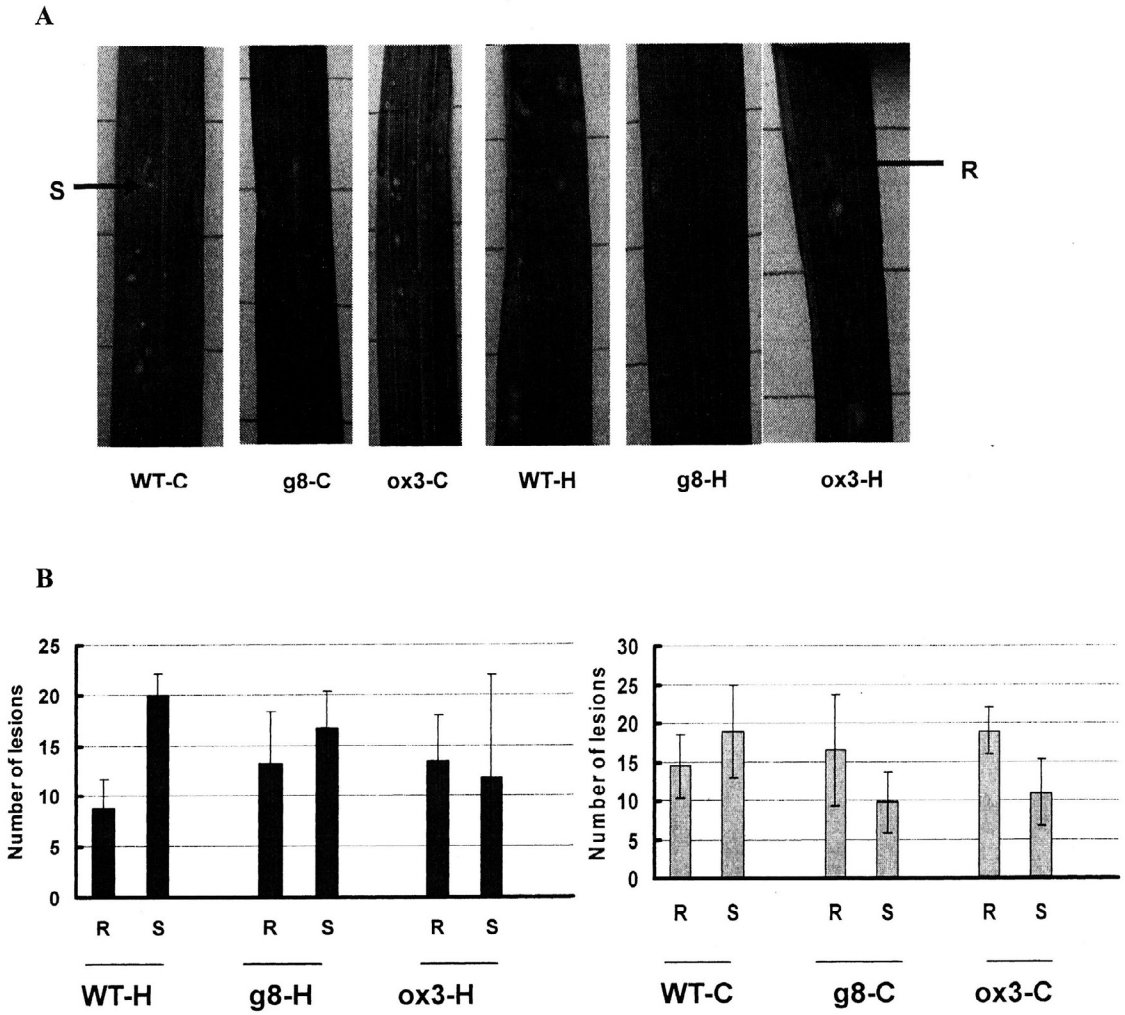

Fig. 10 A, Disease symptoms on the leaves of wild-type and transgenic plants with heat treatment (H) or with no-heat treatment (C). Disease symptoms were characterized 7 days after inoculation. B, The number of lesions on plants grown in a greenhouse. The bar represents the number of susceptible-type lesions (S) and resistant-type (R) in the 10-cm central region of the leaves of individual T_2 transgenic and WT rice plants. There was no difference in the number of lesions between transgenic and WT plants. Conidia of blast fungus (*Magnaporthe grisea* Cavara, race 007) were suspended in 0.01% Tween 20 at a density of 10^5/mL and sprayed onto rice plants.

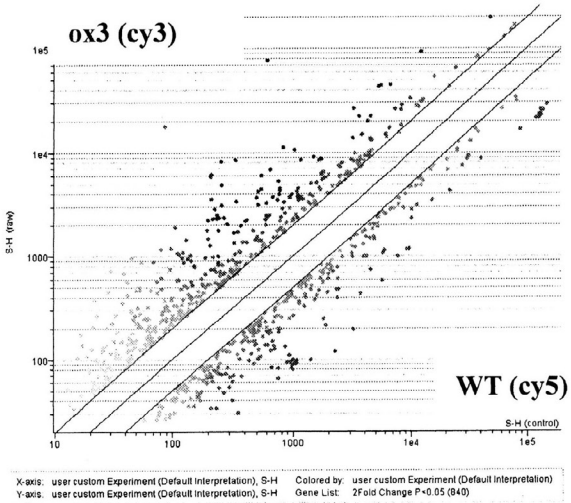

Fig. 11 Gene expression profiles were compared between ox3 and WT with heat treatment at 37/22°C for 2 weeks using 44K Agilent microarray with 3 biological replicates. After heat treatment, 840 genes showed 2-fold or higher changes ($p<0.05$).

Table 3 List of genes up-regulated 10-fold or higher (ox3/WT with heat treatment)

Fold change	Gene	Annotation
122.9	AK120691	Photosystem II 10 kDa polypeptide, chloroplast precursor.
34.1	AK108745	WRKY transcription factor OsWRKY11
28.4	U30477	Alpha-expansin OsEXPA2.
26.6	AK060295	Peroxidase 11 precursor
22.0	Os09g0425200	HMW kininogen family protein.
18.7	AK106292	Basic helix-loop-helix dimerisation region bHLH domain containing protein.
16.8	AK062353	Hypothetical protein.
15.8	CI405772	Alpha/beta hydrolase family protein.
15.0	AK120987	Cytochrome P450 family protein.
14.8	Os04g0429600	Cellulose synthase CesA-1.
14.6	AK103929	Beta-expansin EXPB4 (EXPB4).
14.4	CI187857	Transferase family protein.
12.9	AK062463	Nonspecific lipid transfer protein.
12.7	AK100179	Alpha-expansin OsEXPA4.
12.5	Os07g0131100	Protein kinase family protein.
12.4	AK104883	Lipolytic enzyme, G-D-S-L family protein
12.4	AU096913	Cytochrome P450-like protein (CYP86B1).
11.3	AK072862	Peroxidase (EC 1.11.1.7)
11.3	AK061878	Cytochrome P450 family protein.
11.1	AY177696	Transcription factor MADS27.
11.0	AK105318	Beta-expansin precursor.
10.9	AK104394	Lipolytic enzyme, G-D-S-L family protein.
10.4	AK060241	Conserved hypothetical protein.
10.1	CI066566	Protein kinase domain containing protein.

Table 4 List of genes down-regulated 0.1-fold or lower (ox3 / WT with heat treatment).

Fold change	Gene	Annotation
0.07	AK072692	Apyrase precursor (EC 3.6.1.5) (ATP-diphosphatase) (Adenosine diphosphatase) (ADPase)
0.07	CI355842	Nucleoside phosphatase GDA1/CD39 family protein.
0.07	AF088220	*ent*-kaurene oxidase (EC 1.14.13.78) (AtKO1) (Cytochrome P450 701A3).
0.07	AK070585	Short-chain dehydrogenase/reductase SDR family protein.
0.08	AK104390	RNase S-like protein.
0.09	AF030167	Beta-1,3-glucanase precursor

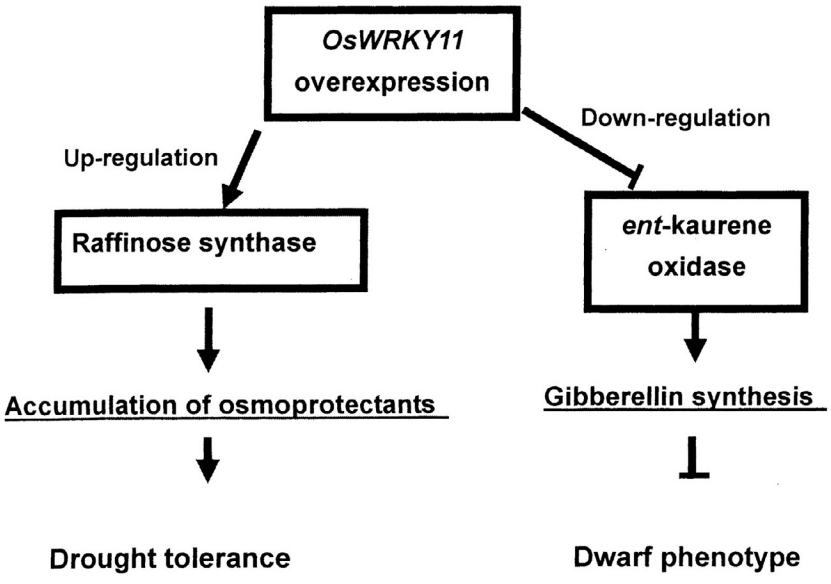

Fig. 12 An ABA-independent novel pathway of drought stress response and tolerance in which *OsWRKY11* plays a role through accumulation of osmoprotectants.

論文審査結果要旨

ストレスで発現誘導される転写因子の遺伝子を用いて、ストレス耐性の組換え植物をつくる試みがなされている。WRKY ドメインをもつ転写因子は耐病性に関与することが報告されているが、*WRKY* 遺伝子を過剰発現させた植物が高温／乾燥ストレス耐性を示すかどうかの知見はない。本研究では、高温と乾燥処理で発現誘導される *OsWRKY11* に注目し、*OsWRKY11* を過剰発現させたイネの解析を行い、高温／乾燥ストレスを評価するとともに、遺伝子発現の解析を行ったものである。

プロモーターとして用いたヒートショックタンパク質 HSP101 のプロモーターがヒートショックで発現誘導されることを確認し、37 度で処理すると *OsWRKY11* の発現が誘導されることを明らかにした。また、*OsWRKY11* を過剰発現させると植物が矮性になることを示した。幼苗に高温／乾燥処理を行い、萎れの程度と回復、緑色部割合を評価し、*OsWRKY11* を過剰発現させた系統が高温／乾燥耐性を示すことを明らかにした。また、切り取った葉の重量の時間経過を測定することにより、乾燥耐性を示すことを明らかにした。さらに、糖類含量の分析を行い、ラフィノースが蓄積していることを見出した。マイクロアレイ解析を行って、遺伝子発現を網羅的に解析したところ、ラフィノース合成酵素遺伝子の発現が増加していることを明らかにした。これより、*OsWRKY11* がラフィノース合成酵素遺伝子の発現を増加させ、ラフィノースが蓄積して浸透圧を高く維持することにより、乾燥耐性を獲得したと考察できた。従来、乾燥耐性には、アブシジン酸(ABA)を介した経路や ABA を介さない経路として転写因子 *DREB* が関わるものが知られていた。しかし、*OsWRKY11* 過剰発現体では、ABA 処理と *DREB* 過剰発現で発現誘導される遺伝子群が発現誘導されていないことを見出した。従来知られていない新規な経路を新たに提唱することができた。

以上のように本研究では、*OsWRKY11* を過剰発現させたイネが、従来知られていない新規な経路により乾燥耐性を示すことを明らかにし、乾燥ストレス反応に新たな知見を与えた。さらに、HSP101 プロモーターと *OsWRKY11* 遺伝子の育種的有用性を示したものであり、環境適応植物工学に多いに貢献するものである。よって審査員一同は、本論文は博士（農学）の学位を授与するに値するものと判断した。